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Original Article

Comparative analysis of human telomerase reverse transcriptase mutation in transitional epithelial cell bladder cancer in high and low grade tumors

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ABSTRACT

Aims: We aimed to evaluate the presence of telomerase reverse transcriptase (TERT) promoter region mutations in transitional epithelial cell bladder cancer by analyzing the h-TERT mutation in high and low-grade tumors and to compare it with the clinicopathological data of the patients.

Methods: A total of 90 patients diagnosed with bladder cancer, 60 with low-grade tumors and 30 with high-grade tumors were included in this study. To detect mutations in the TERT gene in the DNA samples obtained, the most frequently mutated region of the gene was amplified by PCR using forward and reverse primers and mutation analysis was performed by sequence analysis.

Results: TERT promoter mutation was positive in 38 and negative in 22 out of 60 patients in the low-grade group, and positive in 26 and negative in 4 out of 30 patients in the high-grade group. Patients with high-grade tumors were 1.940 times more likely to be TERT promoter mutation positive than low-grade patients (p=0.027).

Conclusion: When interpreted together with other studies that have obtained significant results that TERT mutation analysis can be used in diagnosis, tumor grading and follow-up, it is thought that it can be used in the grading and follow-up algorithm.

Keywords: Bladder cancer, telomerase activity, TERT mutation

INTRODUCTION

Bladder cancer is the most common cancer of the urinary tract.[1] Its prevalence increases with age and is influenced by individual or environmental factors such as gender, race, genetics, smoking, industrial chemical exposure, bladder infection, bladder stones or catheterization. It is predicted that the incidence will continue to increase in the next 10 years worldwide.[1] Considering that approximately 75% of newly diagnosed urothelial bladder cancer cases are detected at the non-invasive stage,[2] the importance of early diagnosis becomes evident.

The diagnosis is made by urine cytologic examination and histopathologic specimens obtained by cystoscopic examination. Histopathologic evaluation is based on the presence of muscle invasion. TNM staging is performed with radiologic imaging and histopathologic data. Stage is the most important independent prognostic marker for progression and overall survival in invasive bladder cancer.[3] While the 5-year survival rate for invasive tumors that have not spread outside the bladder is up to 70%, the survival rate for tumors confined to the inner layer of the bladder has been reported to reach 96%. It is known that most of the cases are detected at these stages.[4]

Many genetic factors play a role in the development of urothelial cancer. Deletion of chromosome fragments, epigenetic alterations, gene mutations and mRNA alterations are the most common disorders that can cause carcinogenesis. These changes become more pronounced with loss of control of the cell cycle, genomic instability and telomere dysfunction.[5,6]

Telomeres are specialized heterochromatin structures located at the ends of linear chromosomes that protect chromosomes from random DNA breakage, preventing their unwanted ends from joining or fragmenting and additionally

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involved in replication and cell proliferation.[7] Telomerase is a ribonucleoprotein DNA polymerase enzyme that prevents the loss of DNA material after the replication cycle by ensuring the formation of "TTAGGG" repeats at the chromosome ends. This activity of telomerase is essential for long-term replicative survival in cells. Mice lacking telomerase showed signs of impaired tissue homeostasis.[8]

Telomerase reverse transcriptase (TERT), known as h-TERT in humans, is one of the subproteins of the telomerase enzyme. Although this enzyme complex is known to be involved in the elongation of the DNA G3' end, its functions have not been fully elucidated.[8] There are data suggesting that it is closely related to cell immortality and cancer formation.

TERT promoter region mutations have been found to be common in many tumor types. These mutations result in an increase in TERT expression and telomerase reactivation by creating new binding sites for many cellular transcription factors. TERT mutation has been reported in bladder cancer cases with a frequency between 53-83% in different studies. These mutations have been found to be more common in non-muscle invasive bladder cancer cases than other genetic mutations considered as risk. In addition, it has been reported to be seen not only in urothelial carcinomas but also in other histologic subtypes.[9-15]

Bladder cancer patients require repeated cystoscopies during diagnosis, treatment and post-treatment follow-up. Although the role of cystoscopy in these processes is very important, it should not be forgotten that it is an invasive, complicated and expensive procedure. Therefore, the search for an alternative non-invasive method continues and some biomarkers are frequently studied for this purpose. The frequency of TERT mutations up to 80% in bladder cancer suggests that it may become a tool and target in follow-up and treatment. In our study, we aimed to analyze the h-TERT mutation in high- and low-grade tumors in bladder cancer with variable epithelial cells, to elucidate its relationship with tumor grade and to compare it with clinicopathological data.

METHODS

Ethics

This study was conducted in accordance with the decisions of the Declaration of Helsinki and the patient rights regulation, with the approval of the University of Health Sciences Hamidiye Clinical Researches Ethics Committee (Date: 06.09.2021, Decision No: 4).

Patient Selection

This study included 90 patients diagnosed with bladder cancer who were followed up in the Urology Department of Sultan 2. Abdülhamid Han Training and Research Hospital between 2016 and 2021. 60 patients had low grade tumors and 30 patients had high grade tumors. Our study was performed by taking 10 µm thick tissue sections from paraffin blocks in the archive of the pathology department and applying the following procedures.

Mutation Analysis

TERT gene promoter region mutations were analyzed by PCR-based direct sequencing method (sanger sequencing). DNA was extracted from dissected tumor sections after standard deparaffinization using QIAamp DNA FFPE tissue kit (catolog no: 56404) (QIAGEN, Hilden, Germany). To detect mutations in the TERT gene in the DNA samples, HotStarTaq DNA polymerase kit (catolog no: 203205) (QIAGEN, Hilden, Germany) and forward (CAGCGCTGCCTGAAACTC) and reverse (GTCCTGCCCCCCTTCACCTTT) primers, PCR mixtures were prepared in sterile biocabins with a final volume of 50 µl and the most frequently mutated region of the gene was amplified by PCR in a thermal cycler (ABI, Applied Biosystems, USA) and mutation analysis was performed by sequence analysis. PCR mixture consisted of 5 µl 10xPCR buffer, 10 µl Q solution, 1.5 µl dNTP mixture (10 mM), 14 µl primers (7 µl fwd + 7 µl rev) (4 pmol/µl), 0.25 µl HotStartTAq DNA polymerase, 1 µl DNA (50 ng) and distilled water. PCR conditions consisted of a cycle of activation at 95˚C for 15 min followed by denaturation for 30 s, primer annealing at 55˚C for 30 s, chain extension at 72˚C for 42 cycles for 45 s, and final extension for 1 cycle for 10 min, followed by removal to +4˚C for electrophoresis. PCR products were loaded onto a 1.5% agarose gel stained with ethidium bromide (Et-Br) and electrophoresed. The samples were then examined in a UV-transluminator with a wavelength of 312 nm and recorded in a gel imaging system. After it was observed that the samples were PCR amplified to the expected length, the control was working and there was no contamination, the purification of the PCR products was started chain extension at 72˚C for 45 s, final extension for 1 cycle for 10 min, and then removal to +4ºC for electrophoresis.

PCR products were purified for sanger sequencing using QIAquick PCR Purification Kit (catalog #: 28106) (QIAGEN, Hilden, Germany) by adding binding buffer, placing in spin column, washing with spin column wash buffer and eluting in a 1.5 ml tube. Purified PCR products were sequenced bidirectionally (forward and reverse) with BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) according to the manufacturer's protocol on an ABI-3730 (48 capillary) DNA sequencer (Applied Biosystems, USA). Samples were denatured at 95˚C for 3 minutes and immediately cooled rapidly on ice. The plate was then loaded into an ABI-3730 (48 capillary) DNA Analyzer (Applied Biosystems, USA) after appropriate programming. Forward and reverse sequence electrophoregrams were analyzed using SeqScape Software v3.0.

Statistical Analysis

All data analyses were performed with SPSS version 20.0 (SPSS Inc., IBM Corp). Compliance with normal distribution was evaluated by Kolmogorov-Smirnov test. Student t test was used for data conforming to normal distribution and nonparametric Mann-Whitney U test was used for comparison of quantitative variables in case of non-normal distribution. Chi-square test was applied for qualitative variables between groups. Survival was tested using the Kaplan-Meier method. Receiver operating curve (ROC) curves were additionally plotted. The p value <0.05 was considered statistically significant. All data were analyzed by rstudio (2022.02.1 Build461). Descriptive statistics (frequency distributions, percentages) were generated, and logistic regression analysis was used as appropriate (**Table**).

RESULTS

This study included 90 patients with bladder cancer who were followed up in the Urology Department of Sultan Abdülhamid Han Training and Research Hospital between 2016 and 2021.

Table. Logistic regression analysis of risks affecting TERT promoter region mutation

Population patients		n=90	OR (95% CI)	p
Recurrence (TERT) mutation (negative/positive)				
Positive				
Risk	Reference is low risk	26		
	High risk	64	1.940 (1.077-3.493)	0.027
Recurrence (TERT) mutation (negative/C228T/C250T)				
Recurrence of TERT status	Reference is negative			
C228T				
Risk	Reference is low risk	35		
	High risk	24	0.265 (0.081-0.867)	0.028
C250T				
Risk	Reference is low risk	3		
	High risk	2	0.273 (0.034-2.188)	0.221

TERT: Telomerase reverse transcriptase, OR: Odds ratio, CI: Confidence interval

60 of the patients were followed up with low grade tumors and 30 with high grade tumors. Out of 60 patients in the low-grade group, TERT promoter mutation was positive in 38 patients and negative in 22 patients. Among the 30 patients in the high-grade group, TERT promoter mutation was positive in 26 patients and negative in 4 patients (**Figure 1**). The TERT mutation rate was 71.1% in all bladder cancer cases. The most common TERT promoter mutations are a single cytosine change to thymine at chromosome 5 base position 1,295,228 (C228T) or less frequently at base position 1,295,250 (C250T) (-124 and -146 bp from the ATG start site, respectively). Of the 26 positive mutations in the high-grade group, 24 were C228T positive and 2 were C250T positive; of the 38 positive mutations in the low-grade group, 35 were C228T positive and 3 were C250T positive.

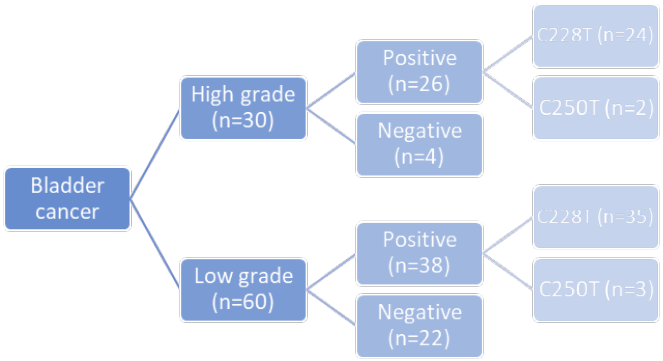

Figure 1. Distribution of bladder cancer cases according to grade and TERT mutations

TERT: Telomerase reverse transcriptase

The mean age of 64 patients who were positive for TERT promoter region mutation (C228T+C250T) was 70.09±10.45 and the mean age of 26 patients who were negative was 68.58±10.61. ROC analysis was performed to determine the cut-off point of the age continuous variable for TERT promoter region mutation. The cut-off point for the mutation analysis of the TERT promoter region mutation was 70, which was not statistically significant [p=0.381; AUC=0.559; 95% CI (0.424-0.694)] (**Figure 2**).

Patients with high grade tumors were 1.940 times more likely to be TERT promoter mutation positive than low grade patients [p=0.027; exp (b) = 1.940 95% CI (1.077-3.493)] (**Table**).

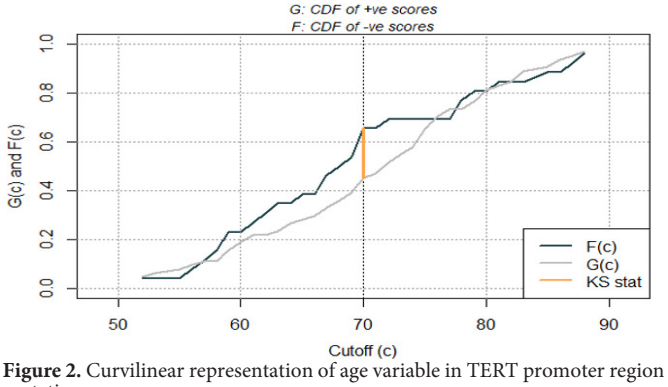

Figure 2. Curvilinear representation of age variable in TERT promoter region mutation

TERT: Telomerase reverse transcriptase

If the patient's bladder cancer is high grade, the probability of TERT recurrence mutation analysis being C228T is 0.265 times higher than if the patient's bladder cancer is low grade [p=0.028; exp (b)=0.265 95% CI (0.081-0.867)], and the probability of being C250T is 0.273 times higher than if the patient has low grade [p=0.221; exp (b)=0.273 95% CI (0.034-2.188)] (**Figure 3**).

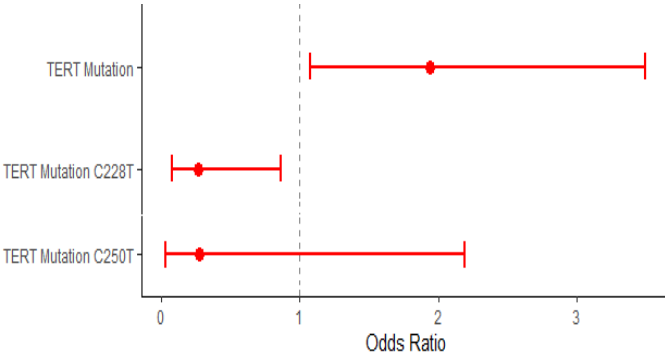

Figure 3. Probabilistic distribution of risks affecting TERT promoter region mutation

TERT: Telomerase reverse transcriptase

DISCUSSION

There is an ongoing search for alternative non-invasive methods to reduce the repeated exposure of bladder cancer patients to an invasive procedure such as cystoscopy and the risks and complications associated with the procedure during follow-up and to intelligently manage increased healthcare costs. Laukthina et al.[16] found that urinary biomarkers can reduce the frequency of cystoscopy by approximately 74% in the follow-up of non-muscle invasive bladder cancer cases.

Based on the discovery of telomerase activity and TERT mutation and the data obtained afterwards, there are studies suggesting that it may be a prognostic marker in various cancer types.[17,18] There is information that hTERT expression is a rate-limiting determinant of the enzymatic activity of human telomerase and that increased expression of hTERT may play a critical role in human carcinogenesis.[19]

It is known that approximately 84% of bladder cancer cases have TERT promoter mutations and the mutant sequence can be detected in urine samples of mutation positive bladder cancer cases.[20-22] Given this high prevalence, TERT promoter mutation analysis would have a high sensitivity in diagnosing bladder cancers. In addition, since these mutations are only seen in cancer cases, it can be predicted that the specificity will also be high.[22] In a study by Pakmanesh et al.,[23] TERT

promoter mutation in urine was found to be 100% sensitive and 88% specific for detecting first diagnosed bladder cancers, while it was 50% sensitive and 88% specific for detecting recurrent bladder cancers. The overall sensitivity and specificity of TERTpm for detecting bladder cancer were 67.7% and 88.0%, respectively, which were consistent across different tumor stages and grades.

In addition to urothelial carcinomas, these mutations have also been reported in other rare histologic variants of primary bladder cancers such as squamous cell carcinoma,[14] small cell carcinoma,[15] non-enteric type adenocarcinoma[13] and plasmacytoid urothelial carcinoma.[11] Furthermore, TERT promoter mutations may serve as biomarkers to differentiate subtypes of urologic malignancies.[12]

Carrasco et al.[24] conducted genetic studies in muscle invasive bladder cancer patients before and after radical cystectomy. In 39 bladder cancer patients, TERT mutation was analyzed in blood samples taken preoperatively and one, four and twelve months postoperatively. TERT c.-124C> T mutation was found to be particularly sensitive in showing tumor recurrence at twelve months. Wang et al.[25] 2015 in Chinese patients, TERT promoter was identified in 47.8% of patients and urine samples were obtained from some of the patients carrying the mutation and analyzed. The mutant promoter was detected in the urine of 52% of patients before the operation and disappeared in most urine samples (80%) examined 1 week after the operation. The results are promising that TERT mutation analysis can be used in postoperative recurrence screening.

In our study, the TERT mutation rate in bladder cancer was 71% and the probability of TERT mutation was 1.94 times higher in cases with high grade tumors, which is consistent with many studies in the literature. Although there are a limited number of studies examining the relationship between grading and TERT mutation in bladder cancer, different results have been obtained in these studies.[20,23,26] Allory et al.[20] reported a TERT promoter mutation frequency of 83% in bladder tumors, independent of stage or disease-related risk. The mutation frequency was almost identical for low-risk non-muscle invasive bladder cancer (NMIBC) (73%) and high-risk NMIBC (74%). Muscle invasive bladder cancer (MIBC) was found to be 53%.[20] In a study by Morozov et al.,[27] the TERT promoter mutation rate in bladder cancer was up to 80% in both tissue and urine, resulting in a sensitivity of 62-92% for primary tumors and 42% for recurrence. Specificity ranged between 73% and 96%, with no correlation with stage. There are also studies indicating that telomerase activity is related to the pathological grade and clinical stage of the tumor, and that telomerase activity is higher in more advanced grade and deep invasive tumors.[26] Our study also supported these data.

In the future, the search for alternative non-invasive methods to cystoscopy is expected to play a key role in the reduction of morbidity and mortality related to bladder cancer with early diagnosis or early recurrence detection, and the results of TERT mutation analysis used in this direction are predicted to provide valuable information in the initial diagnosis and follow-up of recurrence after treatment.

CONCLUSION

Studies are still ongoing to discover a non-invasive method that can achieve similar accuracy to cystoscopy in follow-up. There are studies that have obtained predictions that telomere activity and TERT mutation analysis can be used for cancer diagnosis, grading, typing, recurrence follow-up and evaluation of treatment efficacy in various tissue types. Considering the high prevalence of TERT mutation in bladder cancer, it is thought to be used as a marker in bladder cancer follow-up. In our study, TERT mutation positivity was found to be associated with the presence of high-grade tumors in bladder cancer cases. Accordingly, it is thought that TERT mutation analysis in these patients may affect the follow-up algorithm of the patient. In the light of previous studies, obtaining significant data that TERT mutation analysis can be used in the diagnosis of bladder cancer cases, tumor grading and follow-up of recurrence after treatment indicates that more intensive and numerous studies should be conducted on this subject.

ETHICAL DECLARATIONS

Ethics Committee Approval

The study was carried out with the permission of the University of Health Sciences Hamidiye Clinical Researches Ethics Committee (Date: 06.09.2021, Decision No: 4).

Informed Consent

Because the study was designed retrospectively, no written informed consent form was obtained from patients.

Referee Evaluation Process

Externally peer-reviewed.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Financial Disclosure

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Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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